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pH-sensitive short worm-like micelles targeting tumors based on the extracellular pH

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Abstract

We developed novel photosensitizing drug-carrying worm-like micelles using a pH-sensitive AB_2 miktoarm block copolymer consisting of one methoxy-poly(ethylene glycol) (mPEG) block (A) and two 3-diethylaminopropylated poly(L-lysine) [poly(Lys-DEAP)] blocks (B_2). In particular, the Y-shaped AB_2 miktoarm block copolymer structure mimicking the phospholipid structure enabled the facile prepartion of worm-like micelles. The unique feature of pH-responsive DEAP as a hydrophobic moiety (non-protonated DEAP) at pH 7.4 and a hydrophilic moiety (protonated DEAP) at pH 6.8 modulated acidic pH-activated micellar disintegration. These worm-like micelles serve as a basic transformer whose assembled conformation disintegrates in the acidic milieu (~ pH 6.8) of solid tumors, resulting in improved phototoxicity due to triggered drug release from disintegrated micelles at acidic tumor sites and high-yield generation of cytotoxic singlet oxygen from the photosensitizing drug. As a result, the tumor volume in the nude mice treated with these worm-like micelles was approximately 5.2 times smaller than those treated with free drug. We anticipate that this system will provide great potential for tumor therapy.

Keywords: Worm-like micelle, pH-sensitive, tumor extracellular pH, tumor therapy

Introduction

Versatility in the use of non-spherical micelles has gained tremendous importance in the field of biomedicine.[1-3] In particular, worm-like polymeric micelles, biologically conceived based on filovirus that infects human cells, have been considered as a long circulating drug carrier that is easily assimilated by the human body,[2] and as a new class of smart drug delivery carrier[1-3] that can be tailor-made according to the pharmaceutical need. Recently, several studies have focused on developing potential worm-like drug carrier systems for successful tumor therapy, introducing tumor-recognizable antibodies, and providing satisfactory biocompatibility or biodegradability.[1-5] However, these technological efforts have achieved rather limited success, primarily due to several practical obstacles inherent to hyper-architectural (worm-like structure) fabrication and physiological *in vivo* conditions.[1-5] Furthermore, the worm-like micelles fabricated using conventional amphiphilic polymers have usually exhibited non-aggressive interactions with *in vivo* tumors.[1-5] Their lack of selectivity or specificity of drug release for *in vivo* tumor tissues generally results in relatively low effectiveness. Therefore, the design of functional worm-like micelles that display stimulus responsiveness[6-16] is expected to facilitate the discovery of new opportunities for intelligent drug delivery, high-resolution diagnostics, and site-specific biomedical reactions.

In this study, we introduce a modified poly(L-lysine) [poly(Lys)][17]-based AB_2 miktoarm block copolymer for the facile prepartion of functional worm-like polymeric micelles. In particular, the Y-shaped polymer structure mimicking the phospholipid structure[18,19] was designed for the facile preparation of worm-like polymeric micelles. Recently, Yin et al reported that the Y-shaped amphiphilic block copolymer has a superior capability of forming supra-structural particles compared to the linear amphiphilic block copolymer, although it is not clear how the Y-shaped polymer structure determines the fabricated particle shape.[18] First,

we synthesized methoxy-poly(ethylene glycol) (mPEG, M_w ~ 2KDa) containing a di-hydroxyl group on one side (mPEG-ser) via a simple chemical reaction between the *N*-succinimidyl ester (NSE) group of mPEG and the free amine group of serinol (Supplementary Fig. S1). Then, the di-hydroxyl group of mPEG-ser was di-carboxylated using potassium *tert*-butoxide, ethylbromoacetate, and NaOH/NaCl/HCl (Supplementary Fig. S1). The obtained di-carboxylated mPEG-ser [mPEG-ser-$(COOH)_2$] was coupled with poly(N^{ε}-benzyloxycarbonyl-L-lysine) [poly(Lys-cbz)][17] using *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS), producing mPEG-ser-[poly(Lys-cbz)]$_2$ (Supplementary Fig. S2). The cbz groups of mPEG-ser-[poly(Lys-cbz)]$_2$ were detached using trifluoroacetic acid (TFA)/HBr in acetic acid, yielding mPEG-ser-[poly(Lys)]$_2$. The free pendant amine groups of mPEG-ser-[poly(Lys)]$_2$ were reacted with 3-diethylaminopropyl (DEAP) isothiocyanate, ultimately producing mPEG-ser-[poly(Lys-DEAP)]$_2$ (Supplementary Fig. S2). This AB_2 miktoarm block copolymer, mPEG-ser-[poly(Lys-DEAP)]$_2$, consists of one mPEG block (A) and two poly(Lys-DEAP) blocks (B_2) (Fig. 1a). Here, the conjugation of DEAP (pK_b ~ 7.0)[7] moieties was performed to endow the AB_2 miktoarm block copolymer with pH-responsive properties. The mPEG block can help to avoid any potential immune response.[10-17] In the acidic milieu found in most solid tumors [*e.g.,* human tumor extracellular pH (tumor pH_e),[6-8] which is approximately 6.8], the worm-like micelle fabricated using the AB_2 miktoarm block copolymer is anticipated to provide improved phototoxicity due to triggered drug release (resulting from DEAP protonation-mediated micellar disintegration[7]) and high-yield generation of singlet oxygen from the free photosensitizing drug (Fig. 1b). We expect that these worm-like micelles may facilitate the successful delivery of a photosensitizing drug [model drug: chlorin e6 (Ce6)][6,7] to improve cell entry and tumor inhibition.[20,21]

Materials and methods

Materials

Methoxy-poly(ethylene glycol) (mPEG, M_w: 2kDa), serinol, triethylamine (TEA), dimethyl sulfoxide (DMSO), potassium *tert*-butoxide, toluene, ethylbromoacetate, NaOH, NaCl, HCl, deoxycholic acid (DOCA), *N,N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), N^{ε}-benzyloxycarbonyl-L-lysine, dichloromethane, triphosgene, anhydrous 1,4-dioxane, n-hexane, anhydrous diethyl ether, hexylamine, DEAE Sephadex A-25, ammonium bicarbonate, potassium tetraborate, anhydrous dimethylformamide (DMF), trifluoroacetic acid (TFA), 33% HBr in acetic acid, sodium borate ($Na_2B_4O_7$), 9,10-dimethylanthracene, 3-diethylaminopropyl (DEAP) isothiocyanate, fluorescein isothiocyanate (FITC), *N*-propyl gallate, and glycerol were purchased from Sigma-Aldrich (USA). Chlorin e6 (Ce6) was obtained from Frontier Scientific Inc. (USA). RPMI-1640, fetal bovine serum (FBS), phosphate buffered saline (PBS, pH 7.4), Tris–HCl (pH 8.4), penicillin, and streptomycin were purchased from Welgene Inc. (Korea). *N*-succinimidyl ester mPEG (mPEG-NSE) was synthesized as described in detail in our previous studies.[20]

Polymer synthesis

The *N*-succinimidyl ester (NSE) group of mPEG-NSE (0.2 mmol) was reacted with the free amine group of serinol (1 mmol) in DMSO (20 mL) containing TEA (1 mL) at room temperature for 24 h, yielding mPEG-ser (Supplementary Fig. S1). The solution was filtered, transferred to a pre-swollen dialysis membrane tube (Spectra/Por® MWCO 1K), and dialyzed against deionized water to remove the non-reacted chemicals. The solution was withdrawn from the dialysis membrane tube and freeze-dried for 2 days. The di-hydroxyl groups of mPEG-ser (4 mmol) were reacted with potassium *tert*-butoxide (24 mmol) in toluene (100

mL) at 110 °C for 4 h and then reacted with ethylbromoacetate (24 mmol) at room temperature for 12 h. The resulting solution mixed with excess diethyl ether produced the precipitated product. The dried product was dissolved in 1 M NaOH containing 10 wt.% NaCl, and the resulting solution was mixed with 1 M HCl at room temperature for 4 h (Supplementary Fig. S1), yielding dicarboxylated mPEG-ser [mPEG-ser-$(COOH)_2$]. The solution was extracted using excess dichloromethane and lyophilized after adding excess diethyl ether. The product was again purified via ion-exchange chromatography. After adding the solution (the product in deionized water) to the column (10×100 mm, packed using swollen DEAE Sephadex A-25 in 0.5 M potassium tetraborate solution), the linear ionic gradient of ammonium bicarbonate solution (5-30 mM) was applied to elute purified mPEG-ser-$(COOH)_2$.[18] The production yield of mPEG-ser-$(COOH)_2$ [dividing the amount of the product by the theoretical yield calculated from feeding amount of mPEG] was approximately 78 wt.%.

Next, poly(N^{ε}-benzyloxycarbonyl-L-lysine) [poly(Lys-cbz)] was prepared as described by van Dijk-Wolthuis et al. Briefly, *N*-carboxy-(N^{ε}-benzyloxycarbonyl)-L-lysine anhydride (0.03 mmol), precipitated in excess n-hexane after the chemical reaction of N^{ε}-benzyloxycarbonyl-L-lysine and triphosgene in anhydrous 1,4-dioxane at 50°C for 2 h) was ring-opening polymerized using hexylamine (initiator, 0.001 mmol) in anhydrous DMF (30 mL) at room temperature for 3 days.[6,17] The obtained solution mixed with excess diethyl ether produced the precipitated poly(Lys-cbz).

Then, the mPEG-ser-$(COOH)_2$ (0.02 mmol) dissolved in DMSO (20 mL) was reacted with the terminal amine group of poly(Lys-cbz) (0.08 mmol) in the presence of DCC (0.08 mmol) and NHS (0.1 mmol) at room temperature for 24 h, yielding mPEG-ser-[poly(Lys-cbz)]$_2$ (Supplementary Fig. S2). The solution was filtered, transferred to a pre-swollen

dialysis membrane tube (Spectra/Por® MWCO 8K), and dialyzed against deionized water to remove the non-reacted chemicals and byproducts. The solution was withdrawn from the dialysis membrane tube and was freeze-dried for 2 days. The cbz groups of mPEG-ser-[poly(Lys-cbz)]$_2$ were detached using TFA (5 mL)/33% HBr in acetic acid (5 mL) at room temperature for 30 min,[17] yielding mPEG-ser-[poly(Lys)]$_2$. The solution was mixed with excess ethanol/diethyl ether (50:50 vol.%), filtered, and lyophilized. The production yield of mPEG-ser-[poly(Lys)]$_2$ [when considering feeding amount of mPEG-ser-(COOH)$_2$] was approximately 64 wt.%. Finally, the free pendant amine groups of mPEG-ser-[poly(Lys)]$_2$ (0.06 mmol) were coupled with DEAP isothiocyanate (5 mmol) in DMSO (30 mL) containing TEA (1 mL) at room temperature for 3 days, yielding mPEG-ser-[poly(Lys-DEAP)]$_2$ (Supplementary Fig. S2). The production yield of mPEG-ser-[poly(Lys-DEAP)]$_2$ [when considering feeding amount of mPEG-ser-[poly(Lys)]$_2$] was approximately 71 wt.%. In addition, to prepare the control polymer, DOCA (pH-insensitive moiety, 5 mmol) was reacted with mPEG-ser-[poly(Lys)]$_2$ (0.06 mmol) in DMSO (30 mL) containing DCC (5 mmol) and NHS (5 mmol) at room temperature for 3 days, yielding mPEG-ser-[poly(Lys-DOCA)]$_2$ (Supplementary Fig. S3). Each solution was filtered, transferred to a pre-swollen dialysis membrane tube (Spectra/Por® MWCO 8K), and dialyzed against fresh DMSO (for 2 days) and deionized water (for 2 days) to remove the non-reacted chemicals and byproducts. Each solution was withdrawn from the dialysis membrane tube and freeze-dried for 2 days. The production yield of mPEG-ser-[poly(Lys-DOCA)]$_2$ [when considering feeding amount of mPEG-ser-[poly(Lys)]$_2$] was approximately 69 wt.%.

The chemical structure and number average molecular weight (M_n) of polymers were analyzed using a Bruker 300 MHz NMR Spectrometer (Bruker, Germany). The weight average molecular weight (M_w) of polymers was analyzed using a HPLC system (Waters,

USA) equipped with a 410 differential refractometer and a GPC KF-804 or GPC KF-805 column (Shodex, Tokyo, Japan) at a flow rate of 1.0 mL/min with DMF as the mobile phase at room temperature.

Worm-like micelle preparation

The mPEG-ser-[poly(Lys-DEAP)]$_2$ or mPEG-ser-[poly(Lys-DOCA)]$_2$ (2 mg) was dissolved in volatile dichloromethane (1 mL) in the presence or absence of Ce6 (0.2~1.2 mg) and transferred to a 50 mL round flask. After evaporating the dichloromethane using a rotary evaporator, the thin polymer film coated on the round flask was rehydrated in 2 mL of a $Na_2B_4O_7$ solution (5 mM), followed by sonication at a frequency of 100 kHz for 20 min. Next, the solution was stirred for 30 min at 200 rpm at 37 °C. In addition, to remove non-encapsulated Ce6 (Ce6 aggregates), the micelle solution was filtered using a 1.2 μm syringe filter.

The amount of encapsulated Ce6 was determined by measuring the UV absorbance of the Ce6-loaded micelles dissolved in DMSO at 670 nm.

Characterization of worm-like micelle

The micelles dispersed in PBS (pH 7.4, 6.8, or 6.0) were stabilized at room temperature for 12 h, dropped on a glass slide, and dried *in vacuo* prior to the analysis. The morphology of the micelle samples was examined via field emission scanning electron microscopy (FE-SEM, Hitachi s-4800, Japan).[20] Furthermore, to evaluate the long-term micelle stability, the micelles were exposed to PBS pH 7.4 (150 mM) containing FBS (10 wt.%) and sodium azide (0.05 wt.%) under mechanical shaking condition (100 *rev.*/min) at 37 °C.

The micelle solution was extracted at a given time, dried *in vacuo*, and analyzed via FE-

SEM. The zeta potential change in each micelle sample (0.1 mg/mL, without Ce6) at different pH values (pH 7.4-6.0) was measured using a Zetasizer 3000 (Malvern Instruments, USA). Before the measurement, each sample was stabilized at room temperature for 12 h.[20]

The micelles dispersed in PBS (150 mM, pH 7.4) (0.5 mL) were added to a pre-swollen dialysis membrane tube (Spectra/Por® MWCO 5K), immersed in fresh PBS (150 mM, pH 7.4-6.0) (20 mL), and incubated in a shaking water bath at 100 rpm and 37°C[20] to preserve the sink condition of Ce6. The outer phase was replaced with fresh buffer solution at each time point. The amounts of released Ce6 in each sample were monitored in DMSO/PBS (150 mM, pH 7.4) solution (90/10, vol.%) co-solvent using a UV/visible spectrophotometer at 670 nm.[20]

Cellular uptake analysis

Human nasopharyngeal epidermal carcinoma KB cells (from the Korean Cell Line Bank) were maintained in RPMI-1640 medium containing 1% penicillin-streptomycin and 10% FBS in a humidified standard incubator with a 5% CO_2 atmosphere at 37°C. Prior to experimentation, the tumor cells (1×10^5 cells/mL) grew as a monolayer and were harvested via trypsinization. Then, the tumor cells were suspended in RPMI-1640 medium at different pH values (the pH of each medium was adjusted using PBS and 1 M HCl) and were seeded on well plates and cultured for 24 h prior to *in vitro* cell experimentation.[7,20]

The amounts of Ce6 that accumulated in KB tumor cells due to treatment with micelles were examined using an Axio Imager D2 fluorescence microscope (detecting Ce6 fluorescence, λ_{ex} = 635 nm, λ_{em} = 720 nm, Carl Zeiss, USA). For this experiment, the KB cells (1×10^5 cells/mL) were incubated in the each micelle (equivalent to 10 Ce6 μg/mL) dispersed in RPMI-1640 medium at varying pH values (pH 7.4-6.0) for 4 h. The treated

cells were washed three times with PBS (pH 7.4) and fixed using 1% formaldehyde in PBS for 10 min at room temperature. Each coverslip was mounted on a microscope slide using a drop of anti-fade mounting media (5% *N*-propyl gallate, 47.5% glycerol and 47.5% Tris–HCl, pH 8.4) to decrease fluorescence photo bleaching.[18]

Singlet oxygen generation

The generation of singlet oxygen from each compound was measured using 9,10-dimethylanthracene. Briefly, each micelle (equivalent to 10 μg Ce6/mL) at pH 7.4, 6.8, or 6.0 was stabilized at room temperature for 12 h. The micelles were then mixed with 9,10-dimethylanthracene (20 mM) in PBS (pH 7.4) and illuminated at a light intensity of 5.2 mW/cm^2 using a 670-nm laser source for 10 min.[6,7] It is known that 9,10-dimethylanthracene displays decreased fluorescence intensity as a result of its selective capturing of singlet oxygen.[6,7] The change in the 9,10-dimethylanthracene fluorescence intensity in each sample was measured using a Shimadzu RF-5301PC spectrofluorometer at λ_{ex} of 360 nm and λ_{em} of 380-550 nm. After 1 h, at which point the fluorescence intensity of 9,10-dimethylanthracene had reached a plateau, the change in the fluorescence intensity of 9,10-dimethylanthracene (F_f - F_s), which corresponds to the fluorescence intensity (F_s) of each micelle (containing Ce6) subtracted from the fluorescence intensity of 9,10-dimethylanthracene (without the Ce6-loaded micelles, indicating no singlet oxygen, F_f), was plotted.[6,7] In addition, NIR fluorescence images of the wells containing the micelles in PBS (pH 7.4-6.0) were captured using a KODAK image station (λ_{ex} = 635 nm, λ_{em} = 720 nm; Image Station 4000 MM; Kodak, USA).

***In vitro* phototoxicity tests**

The Ce6 phototoxicity to the KB tumor cells was examined under light illumination

using a 670 nm laser source. The KB tumor cells treated for 8 h with each micelle at pH 7.4, 6.8, or 6.0 were washed three times with fresh cell culture medium (without the micelles), illuminated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min, and then further incubated for 12 h. Cell viability was measured via a Cell Counting Kit-8 (CCK-8) assay.[6-8] In addition, the original cytotoxicity of the micelles was evaluated after 24 h of treatment without light illumination.

Animal care

The *in vivo* studies were performed using 6- to 8-week-old female nude mice (BALB/c, nu/nu mice, Institute of Medical Science, Tokyo, Japan). The nude mice were maintained under the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (Republic of Korea).

***In vivo* animal experiments**

For the *in vivo* animal experiments, the female nude mice were inoculated with KB tumor cells via a subcutaneous injection of 1×10^5 cells suspended in PBS, pH 7.4 (150 mM). When the tumor volume reached 100 mm^3,[6-8] the micelles (equivalent to 2.5 mg Ce6/kg) or free Ce6 (equivalent to 2.5 mg Ce6/kg) were intravenously injected into the KB tumor-bearing nude mice through the tail vein. A KODAK image station was utilized to obtain live photo-luminescence images of the nude mice.[6-8] At 24 h post-injection, the nude mice were sacrificed, and the excised tumor and organ (liver, spleen, lung, kidney, and heart) tissues were analyzed using a KODAK image station.[6-8]

To monitor the *in vivo* accumulation of micelles in the KB tumors, FITC-conjugated PHWM (prepared via the simple chemical reaction of the FITC and the residual free amine groups of the PHWM in PBS) (10 mg/kg) was intravenously injected into KB tumor-bearing

nude mice (tumor size: ~100 mm^3). At 24 h post-injection, the nude mice were sacrificed, and the excised *in vivo* tumor tissue was sectioned using a microtome and analyzed using an Axio Imager D2 fluorescence microscope (detecting FITC fluorescence).

For *in vivo* tumor inhibition analysis, the micelles (equivalent to 2.5 mg Ce6/kg) or free Ce6 (equivalent to 2.5 mg Ce6/kg) were injected intravenously into the KB tumor-bearing nude mice (tumor volume ~ 100 mm^3) via the tail vein. At 12 h post-injection, the tumor site of the nude mice were illuminated at a light intensity of 5.2 mW/cm^2 using a 670 nm NIR laser for 40 min. The tumor volume was calculated according to the formula: tumor volume=length×$(width)^2/2$.[6,7]

Results and discussion

Worm-like micelle preparation

In this study, we synthesized a pH-sensitive AB_2 miktoarm block copolymer. Briefly, pH-sensitive function moiety (DEAP) was coupled with the free pendant amine groups of mPEG-ser-$[poly(Lys)]_2$ {Lys repeating unit:18.6, as estimated based on the ^{1}H-NMR peaks using the integration ratio of the peaks at δ 0.85 [-C**H**$_3$, corresponding to the terminal group of poly(Lys)] and δ 4.25 [-C**H**-, corresponding to the repeating unit of poly(Lys)] (Supplementary Fig. S4)}, resulting in producing mPEG-ser-$[poly(Lys\text{-}DEAP)]_2$ (Supplementary Fig. S2). The degree of DEAP substitution [defined as the number of DEAP moieties per repeating unit of poly(Lys)] in mPEG-ser-$[poly(Lys\text{-}DEAP)]_2$ estimated based on the ^{1}H-NMR peaks using the integration ratio of the peaks at δ 0.9 (-C**H**$_3$, corresponding to the DEAP moieties) and δ 4.2 [-C**H**-, corresponding to the repeating unit of poly(Lys)] was 0.60 (Supplementary Fig. S5). In addition, we conjugated deoxycholic acid (DOCA, as a pH-insensitive hydrophobic moiety for evaluating pH-sensitive DEAP moiety)[20] to the free pendant amine groups of mPEG-ser-$[poly(Lys)]_2$ and obtained mPEG-ser-[poly(Lys-

DOCA)]$_2$ (Supplementary Fig. S3). The degree of DOCA substitution [defined as the number of DOCA moieties per repeating unit of poly(Lys)] in mPEG-ser-[poly(Lys-DOCA)]$_2$ estimated from ^{1}H-NMR peaks using the integration ratio of the peaks at δ 0.55 (-C**H**$_3$, corresponding to the DOCA moieties) and δ 4.2 [-C**H**-, corresponding to the repeating unit of poly(Lys)] was 0.48 (Supplementary Fig. S6). The M_n (from NMR), M_w (from GPC), and molecular weight distribution (M_w/M_n) of mPEG-ser-[poly(Lys-DEAP)]$_2$ were 10,872 Da, 13,312 Da, and 1.22, respectively. The M_n (from NMR), M_w (from GPC), and molecular weight distribution (M_w/M_n) of mPEG-ser-[poly(Lys-DOCA)]$_2$ were 13,912 Da, 15,700 Da, 1.13 respectively.

Next, we fabricated the pH-sensitive worm-like micelles (hereafter denoted as PHWM) using mPEG-ser-[poly(Lys-DEAP)]$_2$ with the Y-shaped polymer structure. Importantly, these worm-like micelles (micelle fabrication yield ~ 89%) were rapidly fabricated via the film-rehydration method[1-3] (sonication for 20 min followed by stirring for 30 min at 37 °C), unlike other worm-like micelle fabrication methods (using AB diblock copolymer) that require a lengthy preparation process at high temperature.[3-5] Second, we prepared the pH-insensitive worm-like micelle (hereafter denoted as WM) (micelle fabrication yield ~ 85%) via the same film-rehydration protocol (sonication for 20 min and stirring for 30 min at 37 °C) using mPEG-ser-[poly(Lys-DOCA)]$_2$. Here, WM was used as the control group to evaluate the pH-responsive properties of PHWM.

pH-sensitive properties of PHWM

Fig. 2 shows the physicochemical characteristics of the worm-like micelles. As the pH of solution decreased from pH 7.4 to 6.0, the zeta-potentials of the PHWM changed from 0.9 mV to 8.8 mV. In particular, the zeta potentials of the PHWM further increased from 0.9 mV

to 6.0 mV when the pH was reduced from 7.4 to 6.8 (tumor pH_e) (Fig. 2a), indicating the protonation of DEAP at a slightly acidic pH (pH 6.8-6.0). However, no apparent zeta potential change was detected for the WM. Interestingly, the pH-responsive event of PHWM strongly influenced the morphology of these micelles. FE-SEM (Fig. 2b) revealed that the majority of the PHWM exhibited a nearly short worm-like shape at pH 7.4 but became destabilized at pH 6.8-6.0 and partially disintegrated due to the destabilization of micelles modulated by the protonation of DEAP in the micellar core.

Furthermore, drug release rate from the PHWM was also stimulated by a slightly acidic pH. The cumulative drug (Ce6) release rate graphed as a function of pH revealed that the release rate of Ce6 from PHWM at pH 6.8 was faster than that at pH 7.4 (Fig. 3a). After incubation of 24 h, 32 wt.% or 70 wt.% of encapsulated Ce6 was released from the PHWM at pH 7.4 or pH 6.0, respectively. However, the amounts of Ce6 released from the WM at pH 7.4-6.0 were not remarkably different (Fig. 3b). Fig. 3c shows fluorescent images of KB tumor cells treated with the worm-like micelles. Here, treatment with the PHWM resulted in the highest cellular Ce6 uptake at pH 6.8-6.0. The high Ce6 uptake in KB cells was likely due to the increased free Ce6 (resulting from micellar disintegration) and the protonated PHWM that can interact with anionic cellular membranes. However, WM presented no significant differences in their tumor cellular Ce6 uptake at pH values of 7.4-6.0.

Photo-activity of PHWM

Fig. 4 shows the photo-activity of the worm-like micelles at varying pH. We evaluated the photo-activity of the micelles under light illumination (5.2 mW/cm^2 at 670 nm for 10 min). The PHWM typically displayed decreased singlet oxygen generation, corresponding to the auto-quenching effect[7] of Ce6 molecules entrapped in the micellar core at pH 7.4. However, the PHWM displayed highly increased singlet oxygen generation at pH 6.8-6.0

(Fig. 4a) as a result of the decreased self-quenching effect due to the release of free Ce6 molecules from the destabilized micelles. In addition, near infrared (NIR) fluorescence images of wells containing PHWM in PBS (pH 7.4-6.0) revealed that the self-quenching event of Ce6 in PHWM at pH 7.4 led to a decrease in the fluorescence intensity, but this reduction in the fluorescence intensity was recovered at pH 6.8-6.0 due to the de-quenching of the Ce6 molecules (Fig. 4a). Consequently, the down-regulated production of singlet oxygen (Fig. 4a) from PHWM at pH 7.4 caused less phototoxicity to KB tumor cells (Fig. 4b), but the enhanced intracellular Ce6 uptake (Fig. 3c) and improved photo-activity (Fig. 4a) of PHWM at pH 6.8-6.0 resulted in significantly increased phototoxicity to KB tumor cells (Fig. 4b). This is comparable to the low phototoxicity results (Fig. 4c) of WM, which displayed poor singlet oxygen generation from Ce6 at pH 7.4-6.0 (data not shown) due to the down-regulation of Ce6 release and the self-quenching of Ce6 molecules. In addition, PHWM (Fig. 4d) or WM (data not shown) prior to light illumination displayed negligible cytotoxicity after incubation in the cultures for 24 h.

In vivo efficacy of PHWM

Fig. 5 shows the *in vivo* efficacy of PHWM administered to KB tumor-bearing nude mice. First, the *in vivo* tumor pH_e in the KB tumor-bearing nude mice was determined using an Orion 98-63 micro-pH electrode equipped with a 16-gauge beveled tip. The *in vivo* tumor pH_e was measured to be around pH 6.78.[6] Then, the micelles were intravenously injected into the KB tumor-bearing nude mice. As shown in Fig. 5a, the PHWM facilitated high resolution *in vivo* photo-luminescence at the KB tumor site compared to the relatively low photo-luminescence of WM or the poor photo-luminescence of free Ce6 at the KB tumor site (due to poor tumor-selectivity). The site-specific destabilization of PHWM (in response to the acidic tumor pH_e environment) and the enhanced cellular uptake of the protonated PHWM

may provide for further extravasation of additional worm-like micelles from the blood stream by lowering the physical barriers to tumor penetration.[21] Fig. 5b shows the fluorescent intensity of the excised organs (tumor, liver, spleen, lung, kidney, heart). As expected, the *in vivo* tumor accumulation of PHWM was quite significant (Fig. 5b). We also found that PHWM (green fluorescence) was highly accumulated at the tumor site *in vivo* (Fig. 5c).

Fig. 6 shows the reduction in tumor volume of KB tumor-bearing nude mice treated only once with the micelles or free Ce6. The data confirmed that the PHWM led to significant growth inhibition of the KB tumors. The tumor volume in the nude mice treated with the PHWM was approximately 5.2 times smaller than those treated with free Ce6. In addition, the change in the body weight of the nude mice treated using PHWM was insignificant (data not shown), indicating no apparent toxicity to the entire body. Of course, a detailed toxicological evaluation of PHWM should be performed.

Conclusions

The micelle containing a Y-shaped polymer structure and a pH-responsive property, may be more useful than any worm-like micelle system that has thus far been developed. The collective results from a series of both *in vitro* and *in vivo* studies strongly support that PHWM improves photodynamic tumor inhibition. We anticipate that this worm-like system can be used as a more effective tumor therapy and can provide an example of a method to design functional worm-like micelles.

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Figure Legends

Figure 1. (a) Chemical structure of mPEG-ser-[poly(Lys-DEAP)]$_2$. (b) Schematic concept of the proposed worm-like micelles.

Figure 2. (a) Zeta potential of the micelles (0.1 mg/mL) at pH 7.4, 6.8, and 6.0 (n=3). (b) FE-SEM images of the PHWM at different pH values.

Figure 3. Cumulative Ce6 release profile from (a) Ce6-loaded PHWM or (b) Ce6-loaded WM at different pH values (n=3). (c) Cellular uptake of Ce6 released from each micelle (equivalent to 10 μg Ce6/mL) in KB tumor cells in RPMI-1640 medium at different pH values (4 h incubation) (red fluorescence: Ce6).

Figure 4. (a) Changes in the 9,10-dimethylanthracene fluorescence intensity ($F_f - F_s$, indicating singlet oxygen generation) of PHWM (equivalent Ce6 10 μg/mL). Near infrared (NIR) fluorescence (λ_{ex} = 635 nm, λ_{em} = 720 nm) images of PHWM in PBS (pH 7.4, 6.8, and 6.0) were inserted in the figure. Phototoxicity and cell viability were determined via CCK-8 assay of KB tumor cells treated with (b) PHWM or (c) WM at pH 7.4, 6.8, or 6.0 (8 h incubation). The cells were illuminated for 10 min at a light intensity of 5.2 mW/cm^2 using a 670 nm NIR laser and then incubated for an additional 12 h (n=7). (d) Cell viability was determined via a CCK-8 assay of KB tumor cells treated with PHWM (no light illumination) (n=7).

Figure 5. (a) *In vivo* non-invasive photo-luminescence imaging of KB tumor-bearing nude mice (tumor size: ~100 mm^3) after intravenous injection of the micelles (equivalent to 2.5 mg Ce6/kg) or free Ce6 (equivalent to 2.5 mg Ce6/kg) via the tail vein. (b) *Ex vivo* images of tumor and organ (liver, spleen, lung, kidney, and heart) tissues extracted at 24 h post-injection of PHWM (equivalent to 2.5 mg Ce6/kg). (c) Fluorescence images of *in vivo* tumor tissues from KB tumor-bearing nude mice (tumor size: ~100 mm^3) treated with FITC-conjugated PHWM (10 mg/kg) (green fluorescence: FITC).

Figure 6. Change in the tumor volume of KB tumor-bearing nude mice based on illumination at 12 h post-injection of the micelles (equivalent to 2.5 mg Ce6/kg) or free Ce6 (equivalent to 2.5 mg Ce6/kg) (n=5).

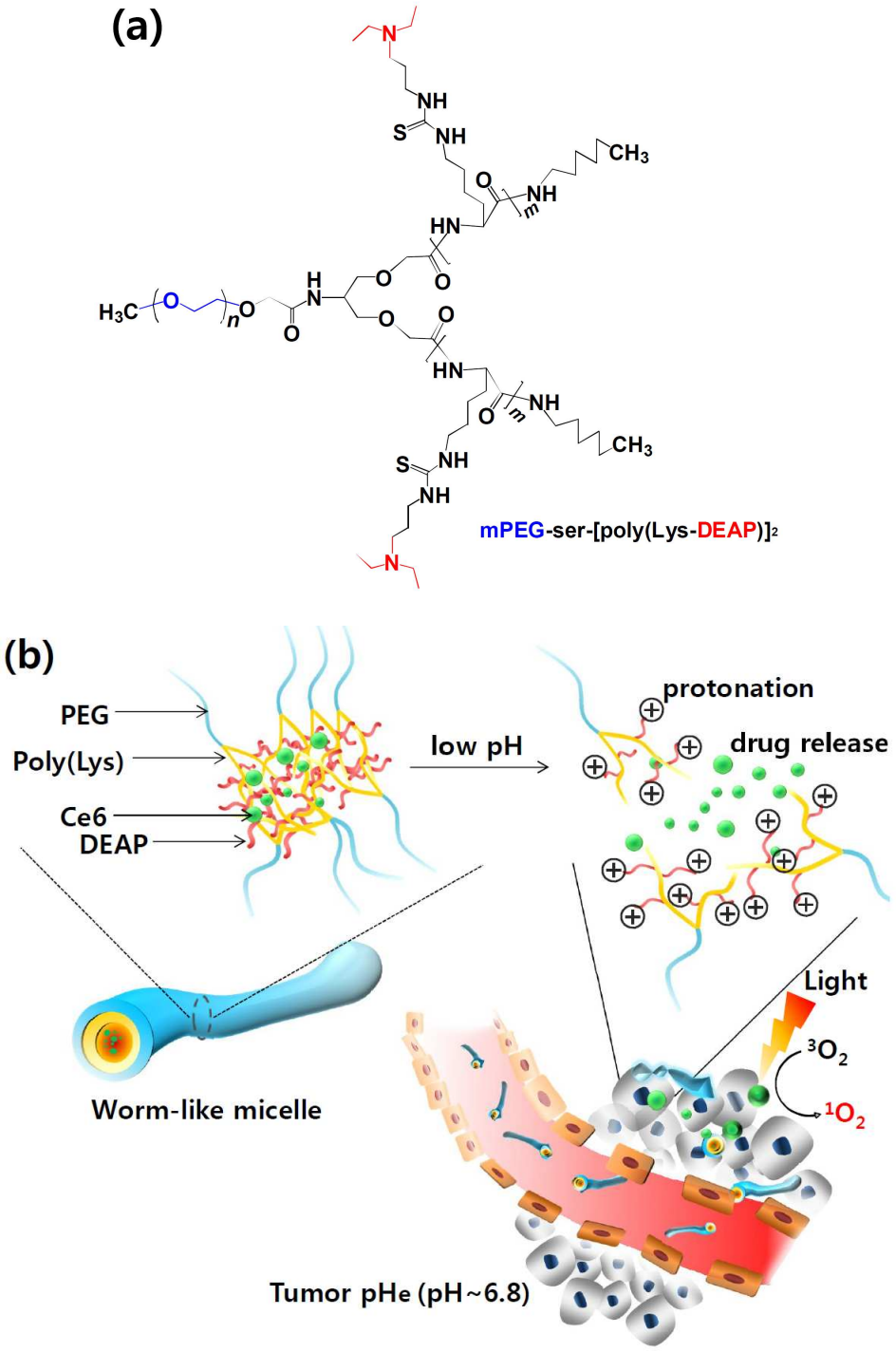


Fig. 1.

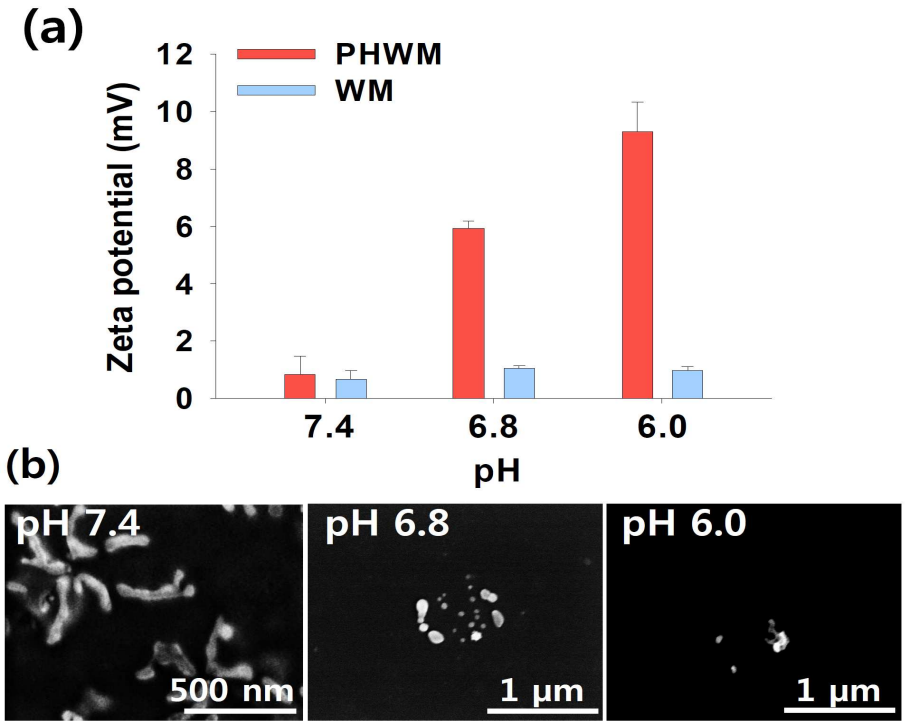


Fig. 2.

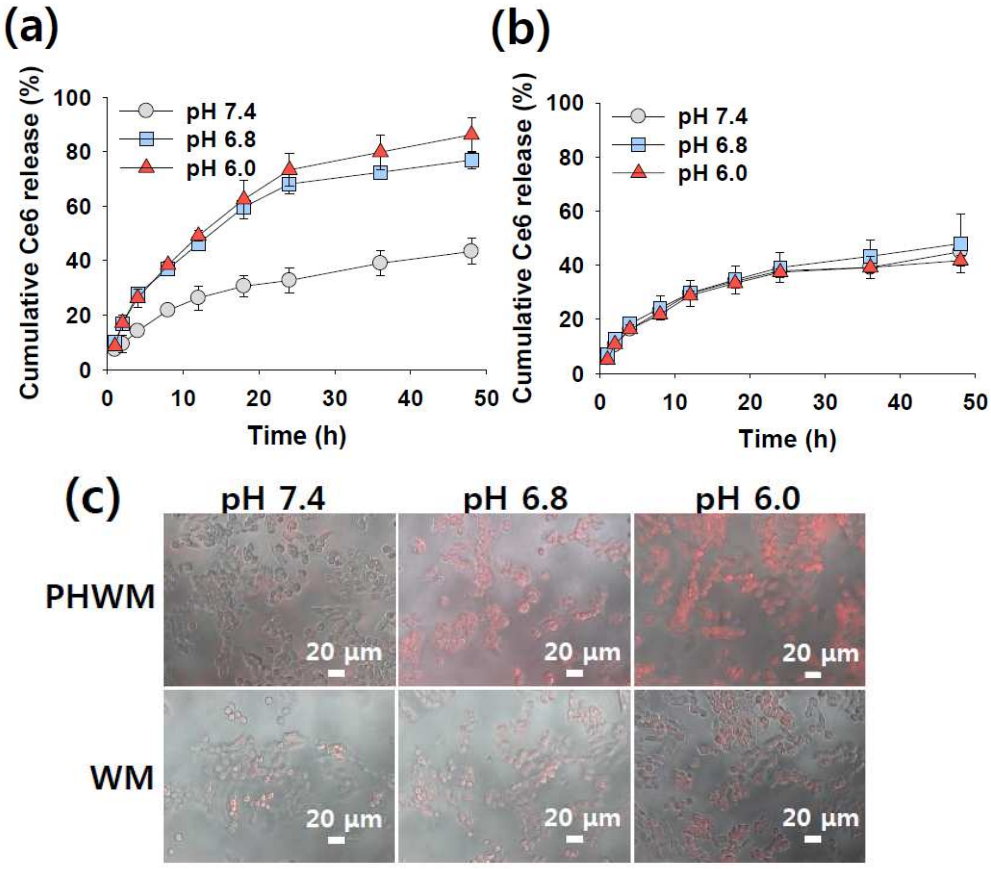


Fig. 3.

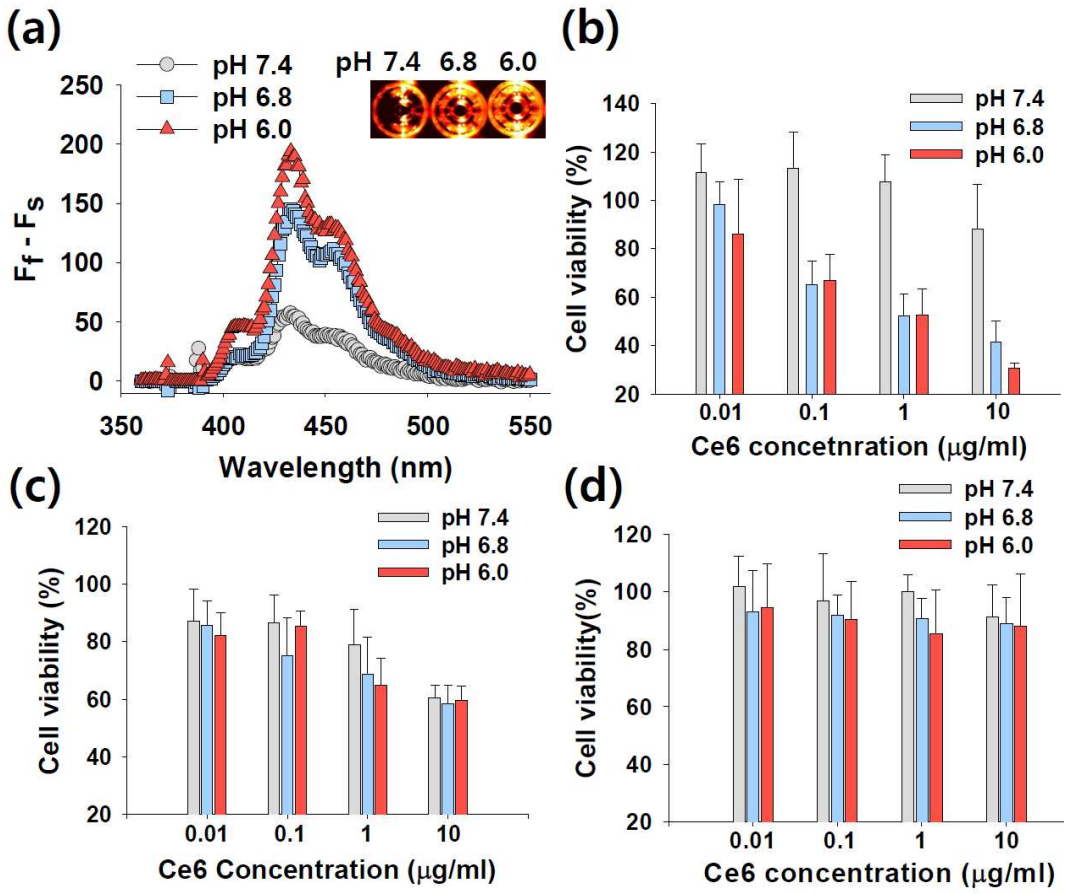


Fig. 4.

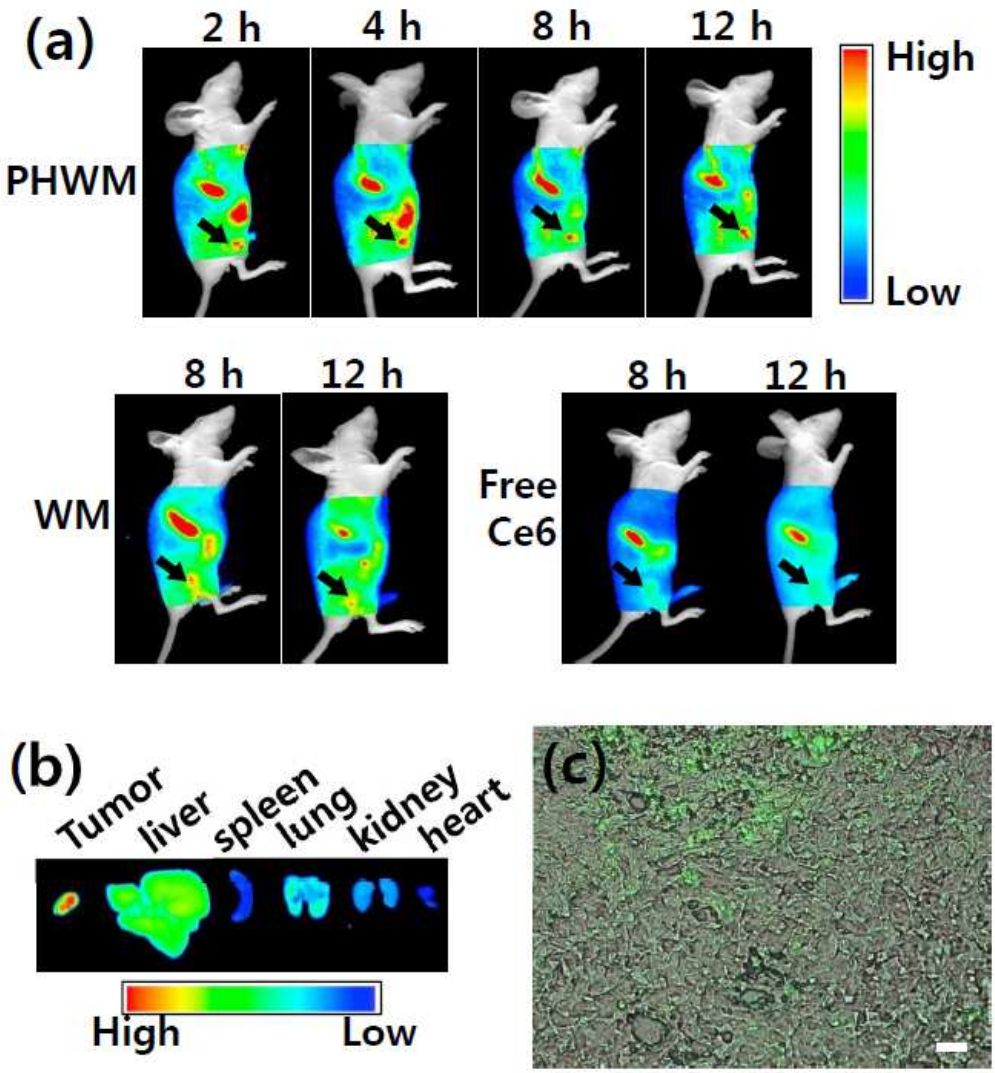


Fig. 5.

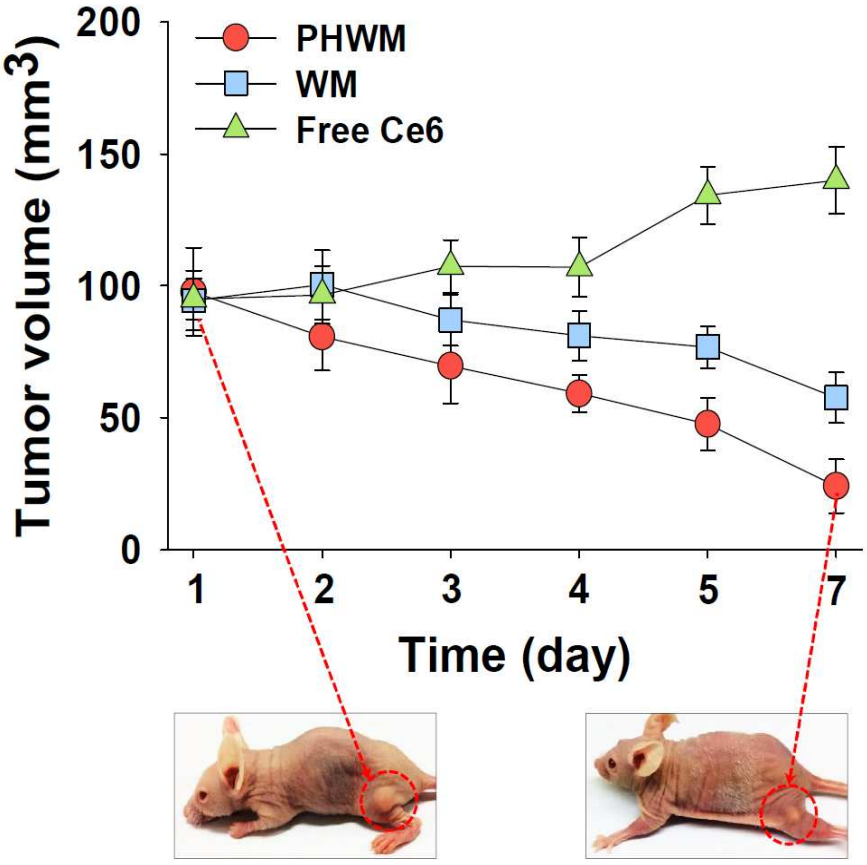
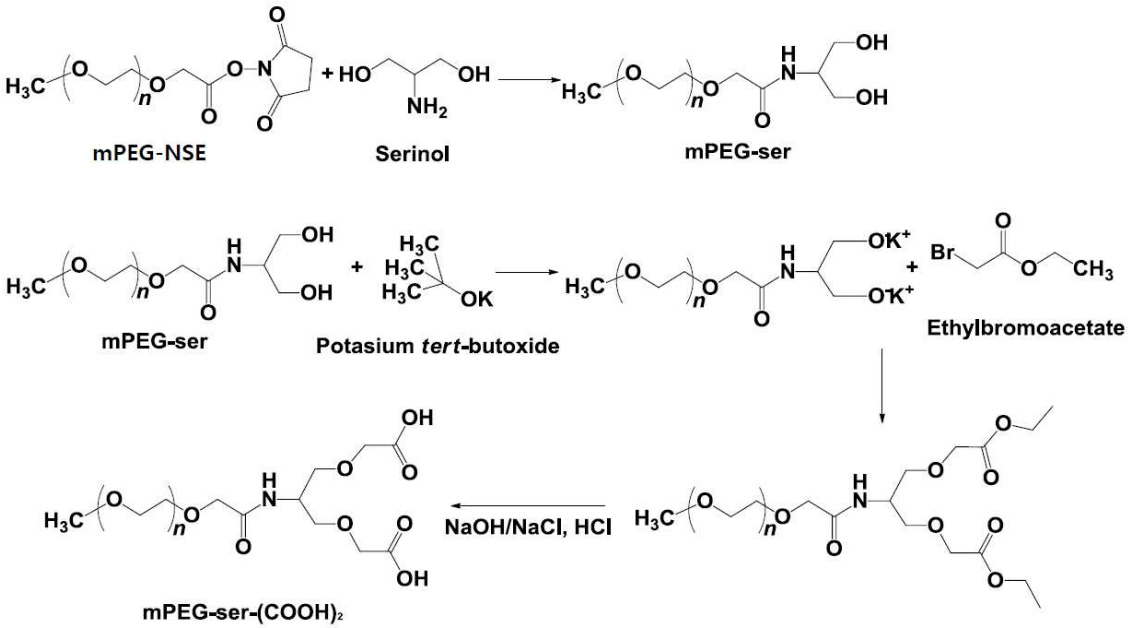


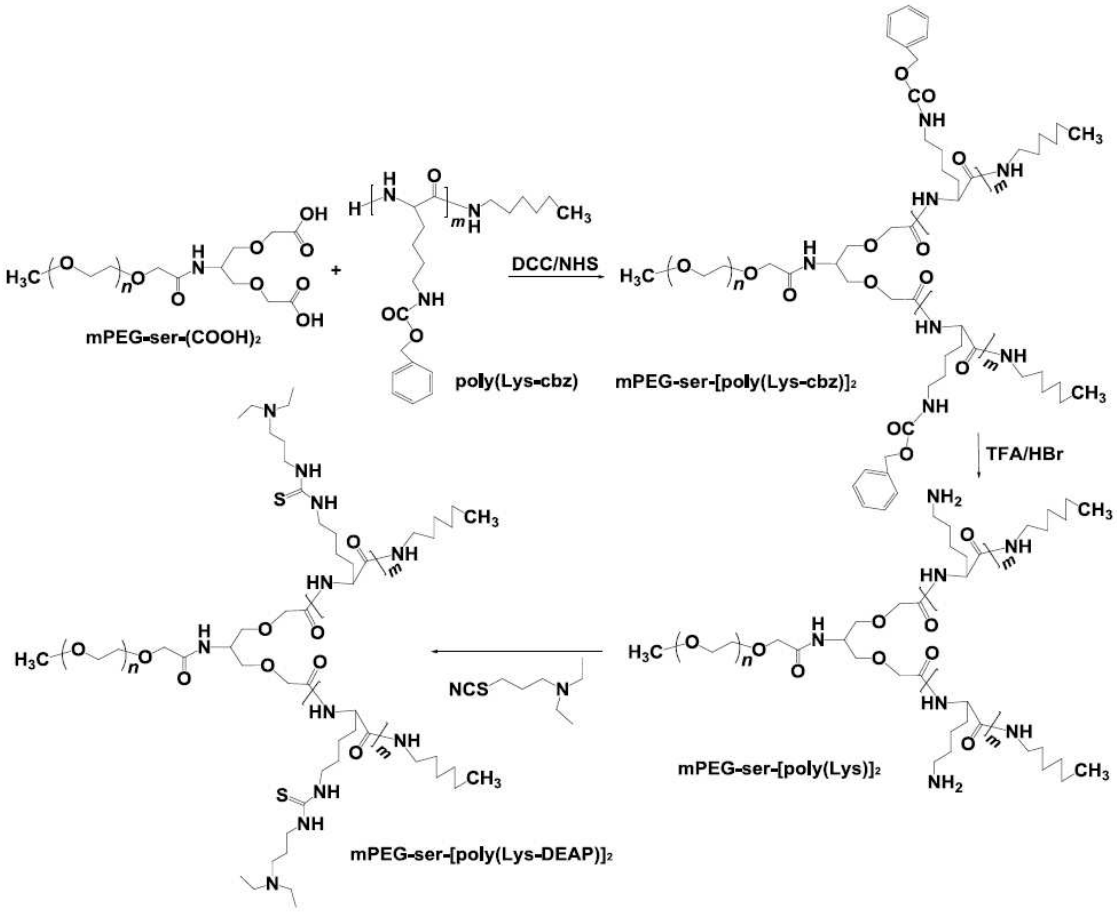
Fig. 6.

Supplementary Information

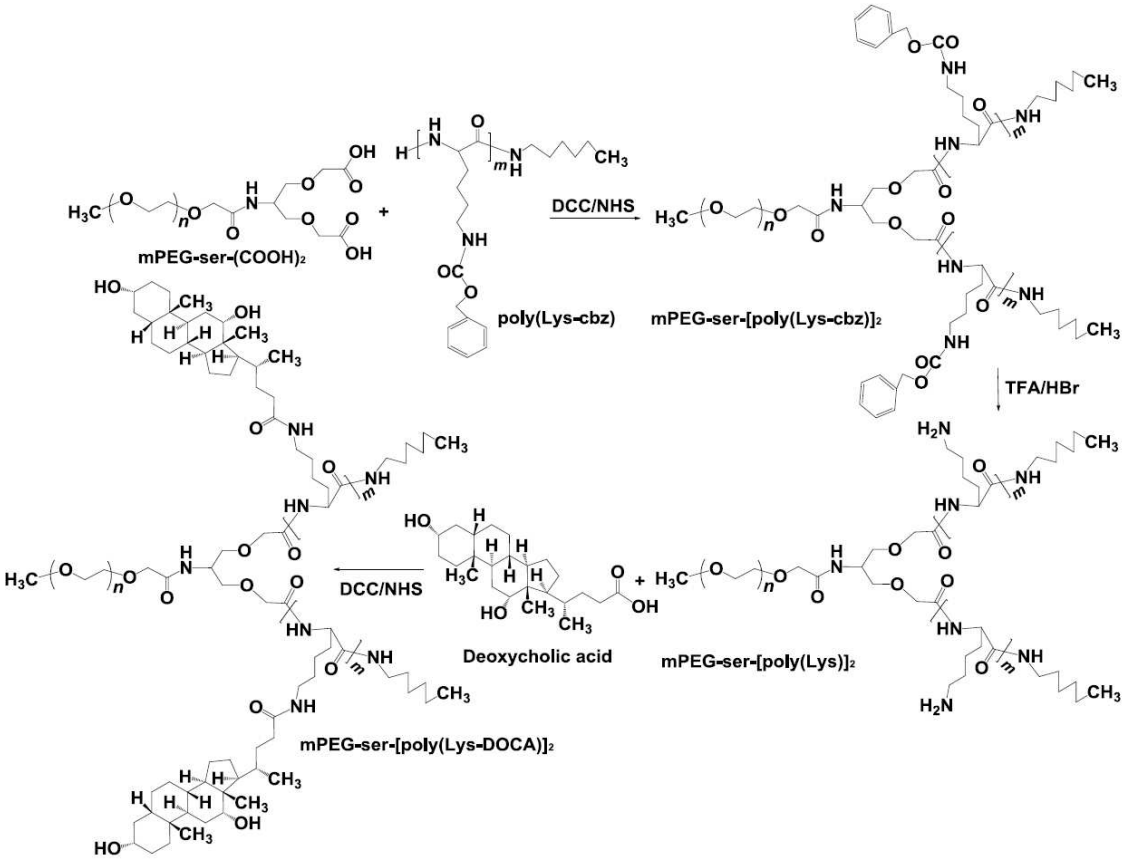
Title: pH-sensitive worm-like micelles for targeting tumor extracellular pH



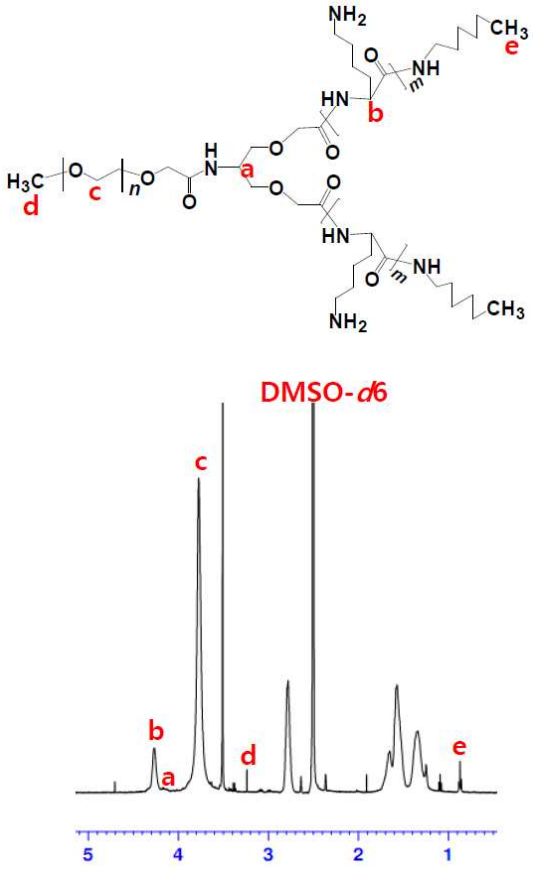
Supplementary Fig. S1. Synthesis scheme of mPEG-Ser-$(COOH)_2$.



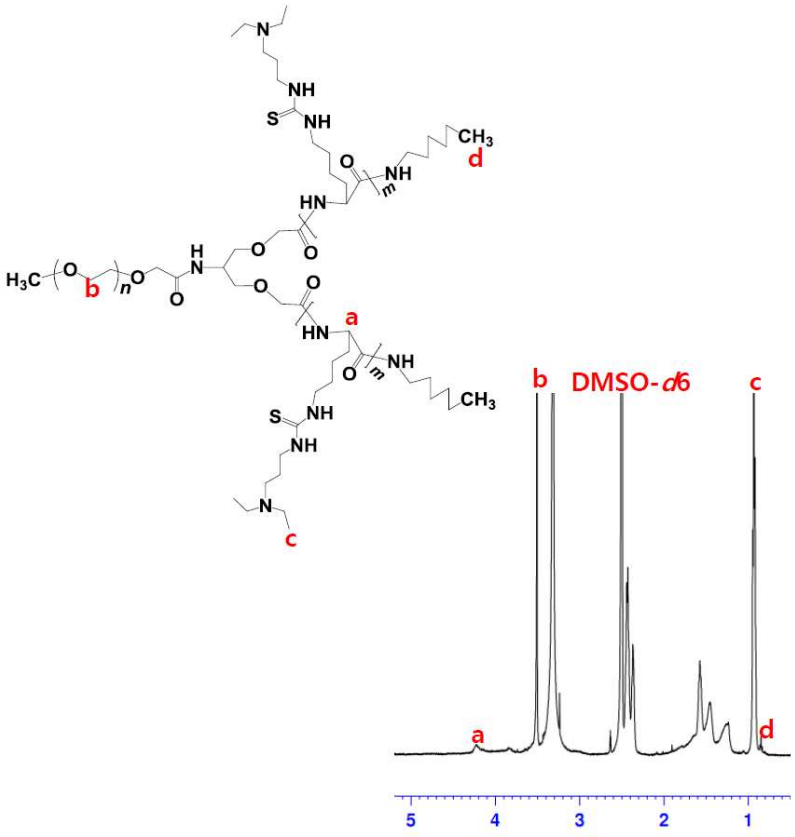
Supplementary Fig. S2. Synthesis scheme of mPEG-ser-[poly(Lys-DEAP)]$_2$.



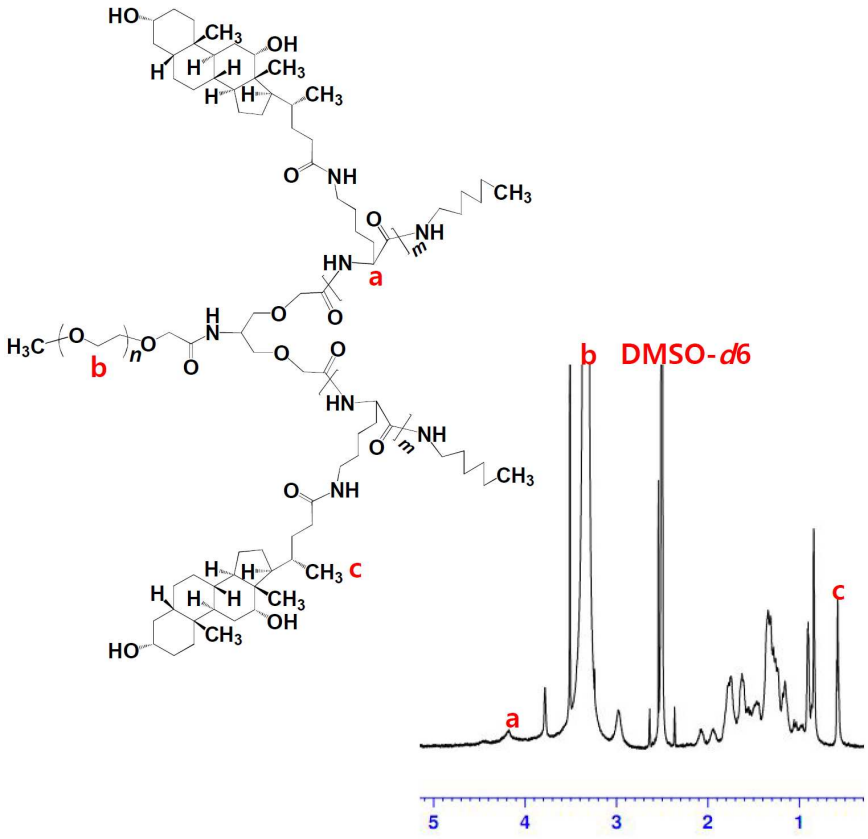
Supplementary Fig. S3. Synthesis scheme of mPEG-ser-[poly(Lys-DOCA)]$_2$.



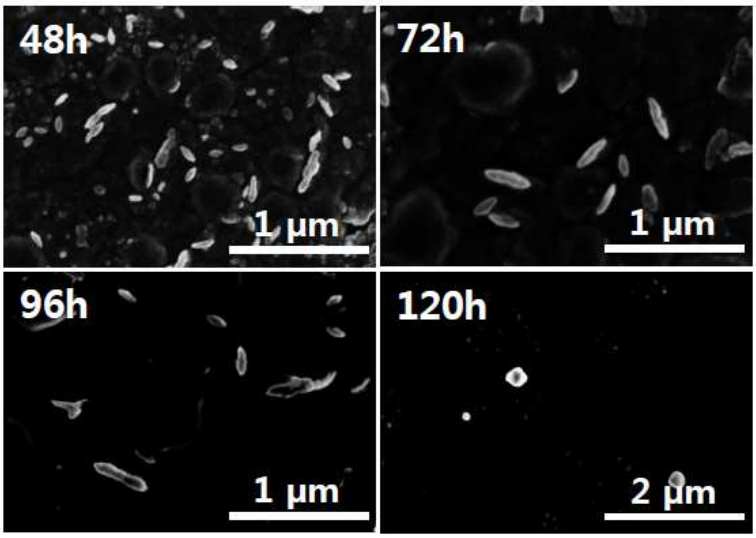
Supplementary Fig. S4. ^{1}H-NMR peaks of mPEG-ser-[poly(Lys)]$_2$.



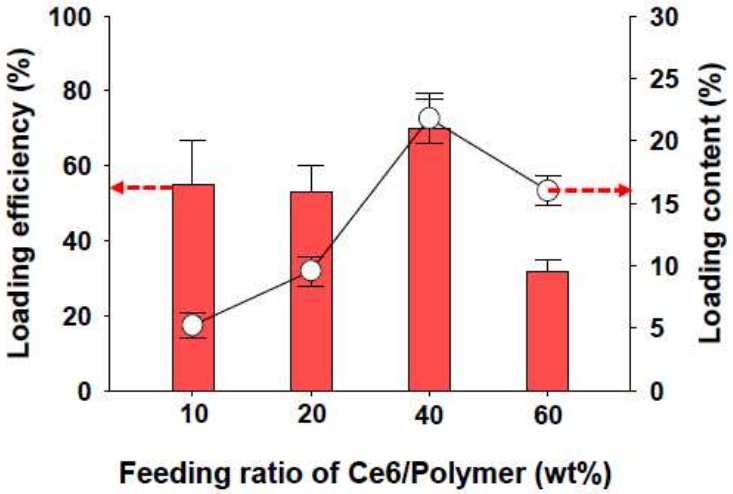
Supplementary Fig. S5. ^{1}H-NMR peaks of mPEG-ser-[poly(Lys-DEAP)]$_2$.



Supplementary Fig. S6. ^{1}H-NMR peaks of mPEG-ser-[poly(Lys-DOCA)]$_2$.

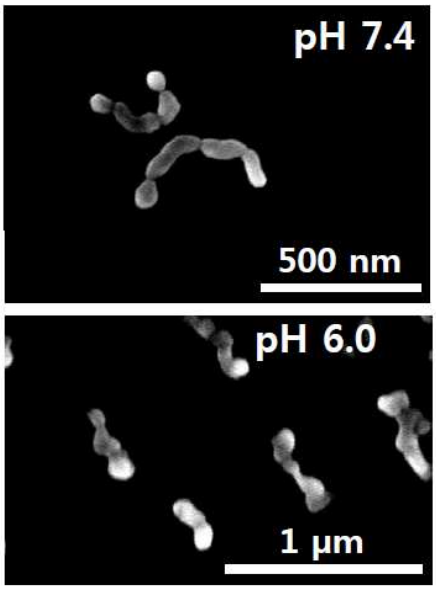


Supplementary Fig. S7. Time-dependent morphological change in PHWM in PBS 7.4 containing FBS (10 wt.%) and sodium azide (0.05 wt.%) under mechanical shaking (100 *rev*./min) at 37 °C. As a result, the PHWM displayed a negligible morphological change over 4 days in serum-containing medium. However, the PHWM underwent fragmentation in 120 h, probably due to the mechanical shear stress (100 *rev*./min) at 37 °C.[2,3]

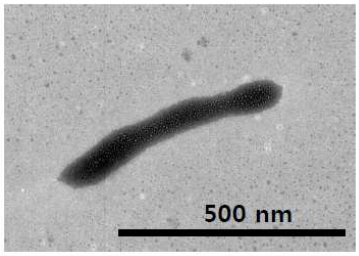


Supplementary Fig. S8. The Ce6 loading efficiency of PHWM based on the feeding ratio of Ce6 (wt.%) to the mPEG-ser-[poly(Lys-DEAP)]$_2$ polymer (n=3). The Ce6 loading efficiency (%) was defined as the weight percentage of Ce6 encapsulated in the micelle relative to the initial feeding amount of Ce6. The Ce6 loading content (%) was defined as the weight percentage of Ce6 entrapped within the micelles relative to the total mass of the Ce6-loaded micelles.

As the Ce6 feeding ratio (wt.%) increased, the Ce6 loading content of the PHWM rapidly increased. However, at a high Ce6 feeding ratio (60 wt.%), the Ce6 loading content of the PHWM decreased, which may have been due to the limited space available to accommodate the Ce6 or quick Ce6 aggregation (precipitation) due to the high concentration of insoluble Ce6.

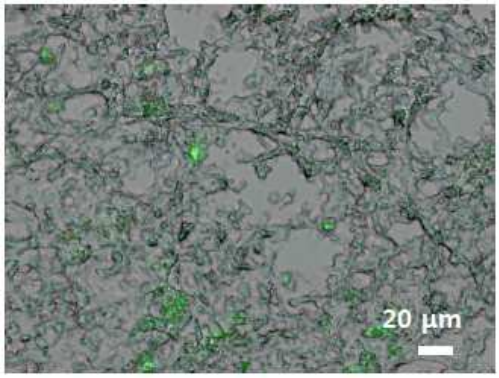


Supplementary Fig. S9. FE-SEM images of the WM at different pH values.



Supplementary Fig. S10. Transmission electron microscope (TEM; JEM 1010, Japan) image of the Ce6-loaded PHWM at pH 7.4.

Here, the morphology of the Ce6-loaded PHWM was confirmed using a transmission electron microscope (TEM; JEM 1010, Japan). The micelles were mounted onto carbon-coated copper grids and examined using a TEM operated at 60 kV and a CCD camera (SC1000 Orion, USA).



Supplementary Fig. S11. Fluorescence images of *in vivo* tumor tissues from KB tumor-bearing nude mice (tumor size: ~100 mm^3) treated with FITC-conjugated WM (equivalent Ce6 10 mg/kg) (green fluorescence: FITC).

Supplementary Table S1. IC50 values obtained on KB tumor cells treated with each sample. The KB tumor cells treated for 8 h with each sample at pH 7.4, 6.8, or 6.0 were washed three times with fresh cell culture medium (without the samples), illuminated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min, and then further incubated for 12 h.

	PHWM			WM			Free Ce6		
	pH 7.4	pH 6.8	pH 6.0	pH 7.4	pH 6.8	pH 6.0	pH 7.4	pH 6.8	pH 6.0
IC50	> 10μg/ml	1.7 μg/ml	1.4 μg/ml	> 10μg/ml	> 10μg/ml	> 10μg/ml	> 10μg/ml	> 10μg/ml	> 10μg/ml